# Determination of Cytokine Responses Using a Multiplexed Fluorescent Microsphere Immunoassay

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## Abstract

*We used a multiplexed fluorescent microsphere immunoassay to develop a sandwich capture assay to assess simultaneously the production of thymus helper (TH) 1- and TH2-type cytokines in tissue culture supernatant obtained from stimulated peripheral blood mononuclear cells. The assay then was used to assess the cytokine production of patients with hyperimmunoglobulinemia E syndrome and in cord blood from neonates. The multiplexed assay has a reportable range of less than 10 to 50,000 pg/mL. For linearity and recovery studies,* $R^2$ *values for the 6 cytokines ranged from 0.988 to 0.999 for samples spiked with known concentrations of recombinant cytokine standards and for patient samples. The assay showed good specificity, with little cross-reactivity between cytokines. Results from supernatants of* Staphylococcus aureus*–stimulated peripheral blood mononuclear cells obtained from 6 patients with hyperimmunoglobulinemia E syndrome showed significantly less interferon (IFN)-gamma production than cells from healthy control subjects. Cord blood cells from neonates produced significantly less interleukin 12 and IFN-gamma than cells from adults in group B streptococci–stimulated mononuclear cells. The fluorescent multiplexed microsphere immunoassay can be used to quantitate multiple cytokines from 1 sample and should be useful for further understanding of the cytokine role in disease.*

Cytokines are produced by a variety of different cell types and are important immunoregulators in the processes of inflammation, hematopoiesis, and wound healing. Individual cytokines can have multiple effects on the growth and differentiation of many cell types and may exhibit considerable overlap with other cytokines in their biologic effects on these cells. The analysis and quantitation of cytokines in biologic fluids and tissue culture supernatant have become widely used in research, constitute an emerging field of interest in clinical laboratory medicine, and clearly are important in furthering our understanding of many immunologic and inflammatory disorders.

In patients with recurrent or severe infections, the possibility of an underlying immunodeficiency should be considered. Although many factors are involved, several studies have shown that decreased or defective cytokine production may have a role in immunologic disorders such as common variable immunodeficiency and hyperimmunoglobulinemia E syndrome.[1,2] Common variable immunodeficiency involves a B-cell defect in the maturation and differentiation process from hematopoietic stem cells to plasma cells and their secretion of immunoglobulins. It is characterized by markedly decreased serum immunoglobulin levels, normal or nearly normal numbers of circulating immunoglobulin-bearing mature B cells, impaired antibody responses, and recurrent bacterial infections of the sinopulmonary tract.[3,4] These B-cell defects often can be associated with deficient secretion of interferon (IFN)-gamma or interleukin (IL)-2.[5] The hyperimmunoglobulinemia E syndrome and associated recurrent infections involve a neutrophil chemotaxis disorder that may be associated with altered T-cell activity and allergic mediators. Patients with chemotactic disorders have manifestations

of recurrent cutaneous abscesses, mucocutaneous candidiasis, and severe pneumonia.[6] Borges et al[2] and Del Prete et al[7] have reported deficient production of IFN-gamma in patients with this disorder. Also included in the present study were group B streptococci–stimulated mixed mononuclear cells obtained from umbilical cord blood of 8 newborns. Neonates have defects in neutrophil function and are deficient in the production of the phagocyte activator IFN-gamma and IL-12, a major enhancer of IFN-gamma.[8-10]

The optimal manner in which to correlate a specific disease process with changes in cytokine levels requires analyzing each sample for multiple cytokines. The most common method for the quantitation of secreted cytokines is the enzyme-linked immunosorbent assay (ELISA). Assays for a variety of cytokines are readily available from numerous commercial vendors. While ELISAs show good sensitivity and specificity, they have many drawbacks in assaying a panel of multiple cytokines in 1 sample. Each ELISA can measure only 1 cytokine per well and requires up to 200 µL of sample per test. When attempting to assay 6 or 7 cytokines, sample volume limitations become an issue, especially when working with tissue culture supernatant. Even with assays from the same manufacturer, sample dilutions, labeled antibodies, and incubation times vary greatly between tests, making it difficult to run several assays at once. Since cytokine production by patients' cells varies greatly, the dynamic range of only 2 to 3 logs of the ELISAs requires that many samples must be diluted and retested. By using a multiplexed fluorescent microsphere immunoassay system (Luminex, Luminex, Austin, TX), we developed a sandwich capture assay to simultaneously assess the production of TH1-type (IFN-gamma, IL-2, and IL-12) and TH2-type (IL-4, IL-6, and IL-10) cytokines in tissue culture supernatant obtained from stimulated mononuclear cells.

The Luminex Multi-Analyte Profiling technology is based on microscopic polystyrene particles called microspheres that are labeled internally with 2 different fluorophores. When excited by a 635-nm laser, the fluorophores emit at different wavelengths, 658 nm and 712 nm. By varying the 658:712 emission ratios, an array of up to 100 different fluorescent profiles have been created. By using precision fluidics, digital signal processors, and advanced optics, the Luminex 100 analyzer classifies each microsphere according to its predefined fluorescent emission ratio. Thus, multiple microspheres coupled to different analytes can be combined with a single sample and assayed. A third fluorophore coupled to a reporter molecule allows quantitation of the analyte that has bound to the microsphere surface.

In this report, we describe the development and validation of this system for the simultaneous quantification of 6 cytokines in tissue culture supernatant. The clinical usefulness of the assay was demonstrated by analyzing TH1- and TH2-type cytokine responses by the mononuclear cells of patients with immunodeficiency syndromes previously reported to have depressed or defective cytokine production. We also assessed the cytokine production by stimulated mixed mononuclear cells from neonates, which have been shown to be deficient in the production of IFN-gamma and IL-12.

## Materials and Methods

### Clinical Samples

Samples included supernatants from *Staphylococcus aureus*–stimulated peripheral blood mononuclear cells of 6 patients with hyperimmunoglobulinemia E syndrome, 1 patient with common variable immunodeficiency, and 12 healthy control subjects. Also included in this study were supernatants from group B streptococci–stimulated mixed mononuclear cells from the cord blood of 8 newborns compared with group B streptococci–stimulated peripheral blood mononuclear cells from 8 healthy adults.

All patient samples included in the study had been used in previous cytokine studies that were reviewed and approved institutionally.

### Tissue Culture Supernatants

Peripheral blood mononuclear cells and mixed mononuclear cell supernatants were derived from standard tissue culture lymphocyte proliferation assays.[11-14] Briefly, lymphocytes were obtained from anticoagulated peripheral blood by Ficoll-Paque density gradient centrifugation. The cells then were counted on a hemocytometer and adjusted to a concentration of $1 \times 10^6$/mL. Cell cultures were set up in 96-well microtiter plates in RPMI 1640 medium containing 10% heat-inactivated pooled normal human serum, antibiotic antimycotic, and L-glutamine. Mitogens (phytohemagglutinin, concanavalin A, pokeweed mitogen) and antigens (heat-killed *S aureus* and group B streptococci) were added in varying concentrations. Cultures were incubated at 37°C with 5% carbon dioxide for 24-, 48-, and 72-hour intervals. After incubation, instead of labeling the cells with tritiated thymidine as in standard proliferation assays, the cells were centrifuged, and the supernatants were removed, divided into aliquots, and stored at –70°C until assayed for cytokine content.

### Coupling of the Microspheres

For the development of the multiplexed panel of 6 cytokines, monoclonal antibodies to human IL-2, IL-4, IL-6, IL-10 (BD Biosciences, San Diego, CA), IL-12 (R&D Systems, Minneapolis, MN), and IFN-gamma (Pierce-Endogen,

Rockford, IL) were used as capture antibodies. The monoclonal antibodies were diluted in phosphate-buffered saline (PBS), pH 7.2, to a concentration of 50 µg/mL and covalently coupled to carboxylated Luminex microspheres using a 2-step carbodiimide reaction.[15] Carboxylated microspheres were activated for 20 minutes at a concentration of 6.25 × $10^6$/mL in PBS, pH 6.1, with 5 mg/mL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) (Pierce-Endogen). Activated microspheres then were washed with PBS, pH 7.2, followed by centrifugation and incubated with the previously described monoclonal antibodies for 1 hour at room temperature on a rocker. The coupled microspheres then were washed twice with PBS, pH 7.2, + 0.05% polysorbate (Tween) 20 (PBST) and resuspended in 1 mL of PBS, pH 7.2, with 0.1% bovine serum albumin and 0.05% sodium azide (PBSB). The microspheres then were incubated for 30 minutes on a rocker to permit blocking of the unreacted sites and stored at 4°C in PBSB. Since the fluorescent dyes contained with the microspheres are light-sensitive, all activation, centrifugation, and incubation steps were done in the dark. Other measures to reduce light exposure, such as wrapping the reaction microtubes in foil and turning off unnecessary light sources, also were used.

### Multiplexed Cytokine Assay

A standard curve for each cytokine was made by mixing known concentrations of recombinant human cytokines IL-4, IL-6, IL-10 (BD Biosciences), IL-2, IL-12 (R&D Systems), and IFN-gamma (Pierce-Endogen) in RPMI 1640 medium. By making 1:5 dilutions in RPMI medium from 50,000 to 0 pg/mL (medium only), an 8-point standard curve was generated. Each standard was run in duplicate. In addition, 3 controls containing high (5,000-20,000 pg/mL), medium (1,000-4,000 pg/mL), and low (20-800 pg/mL) concentrations of all 6 cytokines were run with each assay.

The 6 different monoclonal capture antibody–coupled microspheres were mixed together at a concentration of 1.0 × $10^5$ of each microsphere per milliliter. We added 50 µL of the microsphere mixture to 100 µL of tissue culture supernatant, cytokine standard, or control, resulting in a final concentration of 5,000 of each individual microsphere (30,000 total) per reaction. The cytokine standards, controls, tissue culture supernatants, and microspheres were incubated for 20 minutes at room temperature using a foil-covered, 96-well filter bottom microtiter plate (Millipore, Bedford, MA) on a shaker. The microspheres then were washed 3 times with 200 µL of PBST by vacuum filtration of the microtiter plate. This was followed by the addition of 100 µL of biotinylated polyclonal antibodies to human IL-4, IL-6, IL-10 (BD Biosciences), IL-12 (R&D Systems), IL-2, and IFN-gamma (Pierce-Endogen) to each well of the microtiter plate. The final concentration of the biotinylated detection antibodies ranged from 1 to 3 µg/mL. Following a second 20-minute incubation on the shaker, the microtiter plate was washed as before, and 100 µL of 10 µg/mL of streptavidin-conjugated R-phycoerythrin (Molecular Probes, Eugene, OR) was added to each well. After a 10-minute incubation and final wash, the microspheres were resuspended in 100 µL of PBST. The 96-well microplate containing the resuspended microspheres was placed in a Luminex 100 instrument with an XY platform (automated microtiter plate handler), where the microspheres were counted and analyzed. The amount of cytokine bound to the microspheres by this antibody sandwich technique is determined by the median fluorescence intensity of the reporter molecule phycoerythrin. When excited at 532 nm, phycoerythrin emits at 575 nm. The median fluorescence intensity of the unknown sample then is converted into a value (pg/mL) based on the known cytokine concentrations of the standard curve using a 5-parameter regression formula. Since the analyte specificity and position of each microsphere classification in the array is known, a single fluorescent reporter molecule can be used to measure all 6 cytokine concentrations.

## Results

### Validation of the Multiplexed Assay

Linearity and percentage of recovery of the multiplexed cytokine assay were assessed by spiking known concentrations of recombinant human cytokines in diluted RPMI medium and using actual patient samples containing native cytokines. For the spiked sample, all 6 cytokines were mixed together in concentrations ranging from 6,000 to 30,000 pg/mL. This sample then was serially diluted and run in duplicate in the multiplexed assay containing reagents for all 6 cytokines. The mean observed value was obtained by averaging the duplicate values (pg/mL), while the expected value was calculated from the known starting amount of spiked material. Linear regression analysis then was used to calculate slope, y-intercept, and $R^2$ for observed vs expected values. Percentage of recovery was calculated by dividing the mean observed value by the expected value. The linear regression recombinant spiking results for the 6 cytokines are shown in **Figure 1**. Mean ± SD recoveries for the recombinant cytokines in the multiplex assay were good (104% ± 17%), ranging from a low of 79% for IL-12 to a high of 126% for IL-6 **Table 1**. To ensure that the capture and secondary antibodies used in the assay also recognized native human cytokines, similar studies were done using patient tissue culture supernatants obtained from stimulated

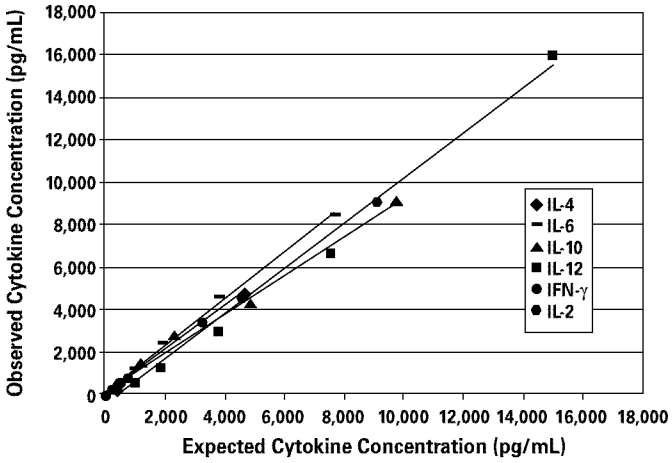


**Figure 1** Linear regression analysis of expected vs observed values (pg/mL) for a single sample spiked with known concentrations of 6 recombinant human cytokines. IFN, interferon; IL, interleukin.

peripheral blood mononuclear cells. Tissue culture supernatant derived from several patients' stimulated peripheral blood mononuclear cells was diluted serially and run in the multiplexed assay. Linear regression analysis and percentage of recovery then was calculated as before. Percentages of recovery for the native cytokines were similar to those for the recombinant cytokines, with a mean ± SD of 101% ± 17%, ranging from 88% for IL-10 to 131% for IFN-gamma (Table 1). A patient sample with sufficiently high concentrations of IL-4 was unavailable and, therefore, was not included in the native cytokine linearity studies.

Sensitivity was determined by measuring the lowest concentration of cytokine that could be differentiated reliably from zero in the Luminex multiplex format. This was determined by statistical comparison of variation of the lowest standard (7.2 pg/mL) with samples containing no analyte.[16] After measuring cytokine concentrations in the RPMI medium blanks in replicates of 10 by 3 different reagent lots, sensitivities at 95% confidence intervals (+ 2 SD) were determined as follows: 5.1 pg/mL for IL-2, 3.4 pg/mL for IL-4, 5.7 pg/mL for IL-6, 4.5 pg/mL for IL-10, 5.2 pg/mL for IL-12, and 6.4 pg/mL for IFN-gamma.

To study the specificity of the monoclonal antibodies coupled to the microspheres in a multiplexed format, high concentrations of only 1 cytokine were spiked into assay diluent to create 6 different samples. These samples then were run in the multiplexed format to check for cross-reactivity of the assay **Table 2**. Some cross-reactivity was seen between IFN-gamma and the other cytokines, most notably with IL-12. In some cases, matched antibody pairs (usually a monoclonal capture antibody and biotinylated polyclonal secondary antibody) available from commercial vendors did not perform optimally in the Luminex platform. Initial development of the IL-2 assay was done using a matched antibody pair and recombinant human IL-2 from R&D Systems. The antibody pair showed a good specific reaction to a sample spiked with 10,000 pg/mL of IL-2 cytokine but had high background at 0 pg/mL of IL-2 cytokine. For this reason, 2 other matched antibody pairs from Pierce-Endogen and BD Biosciences were studied. The 3 different vendors' capture antibodies and biotinylated detection antibodies were cross-tested against each other to find the optimal antibody pair. When the Pierce-Endogen capture antibody was matched with the Pierce-Endogen detection body, background (0 pg/mL) was minimal, but there was also no specific response at 10,000 pg/mL. In fact, the Pierce-Endogen capture antibody did not give a specific response when matched to any of the 3 detection antibodies and, therefore, probably did not recognize the recombinant IL-2 from R&D Systems. Good specific signal/background

**Table 1**
**Summary of Regression Analysis and Percentage Recoveries of the Expected vs Observed Values (pg/mL) for Interleukins 2, 6, 10, and 12, and Interferon-gamma Obtained From a Sample Spiked With Known Concentrations of Recombinant Human Cytokines and From Supernatants Containing Native Human Cytokines***

| Cytokine | Source | Slope | y-Intercept | $R^2$ | Mean ± SD Recovery (%) |
|---|---|---|---|---|---|
| Interleukin | | | | | |
| 2 | Recombinant | 0.99 | 74.9 | 0.999 | 108 ± 8 |
| | Native | 1.11 | −23.2 | 0.993 | 96 ± 10 |
| 4 | Recombinant | 1.03 | 85.7 | 0.997 | 114 ± 11 |
| 6 | Recombinant | 1.11 | 159.6 | 0.998 | 126 ± 8 |
| | Native | 0.97 | −1.8 | 1.000 | 94 ± 4 |
| 10 | Recombinant | 0.92 | 137.2 | 0.998 | 109 ± 14 |
| | Native | 0.88 | 0.7 | 0.999 | 88 ± 4 |
| 12 | Recombinant | 1.06 | −506.6 | 0.993 | 79 ± 14 |
| | Native | 1.04 | −9.1 | 0.997 | 96 ± 6 |
| Interferon-gamma | Recombinant | 1.06 | −27.3 | 1.000 | 97 ± 13 |
| | Native | 1.79 | −170.5 | 0.989 | 131 ± 28 |

* A sufficient sample of native IL-4 was not available.

**Table 2**
**Results of Specificity Studies in Which Six Different Samples Were Spiked With High Concentrations of Only One Cytokine and Assayed in the Six-Cytokine Multiplexed Format**

| | | Luminex* Results (pg/mL) | | | | | |
|---|---|---|---|---|---|---|---|
| Sample | Spiked With | IL-2 | IL-4 | IL-6 | IL-10 | IL-12 | IFN-gamma |
| 1 | IL-2 | 18,905 | 0 | 0 | 0 | 0 | 0 |
| 2 | IL-4 | 0 | 9,546 | 3 | 0 | 0 | 0 |
| 3 | IL-6 | 0 | 0 | 20,163 | 0 | 0 | 0 |
| 4 | IL-10 | 0 | 0 | 0 | 13,753 | 0 | 0 |
| 5 | IL-12 | 0 | 0 | 0 | 0 | 22,562 | 0 |
| 6 | IFN-gamma | 9 | 2 | 15 | 4 | 64 | 12,409 |

IFN, interferon; IL, interleukin.
* For proprietary information, see the text.

ratios were achieved when using the BD Biosciences capture antibody and any of the 3 secondary antibodies, with the best signal/background ratio obtained using the BD Biosciences capture antibody, the biotinylated secondary antibody from Pierce-Endogen, and the recombinant human IL-2 from R&D Systems.

Precision of the 6-cytokine multiplexed assay was determined by intra-assay and interassay studies on 3 different reagent lots using the high, medium, and low assay controls. A lot was defined as independent coupling reactions of the monoclonal capture antibodies to the microspheres and separated preparations of the secondary conjugated polyclonal antibodies. For intra-assay precision, the mean, SD, and coefficient of variation (CV) were calculated from 5 replicates of each level of control. Data from a typical run and lot show CVs of mostly less than 10% **Table 3**. For interassay precision, the mean, SD, and CV percentage were calculated from 2 runs each on 3 different lots, for a total of 6 runs. CVs ranged from less than 15% for the high control to up to 30% for the low control (Table 3).

**Table 3**
**Intra-assay Precision Results for the Six-Cytokine Multiplexed Assay for a Typical Reagent Lot (n = 5) and Interassay Precision Results Obtained From Two Runs on Three Different Reagent Lots (n = 6)***

| | IL-2 | IL-4 | IL-6 | IL-10 | IL-12 | IFN-gamma |
|---|---|---|---|---|---|---|
| Intra-assay results | | | | | | |
| High control | | | | | | |
| Mean | 7,426.3 | 567.0 | 13,668.7 | 15,824.0 | 14,255.5 | 6,473.4 |
| SD | 341.9 | 36.8 | 167.8 | 1211.2 | 280.6 | 383.6 |
| CV | 4.6 | 6.5 | 1.2 | 7.7 | 2.0 | 5.9 |
| Medium control | | | | | | |
| Mean | 321.4 | 25.8 | 659.6 | 862.8 | 718.6 | 587.4 |
| SD | 21.8 | 2.4 | 19.8 | 77.5 | 32.0 | 59.4 |
| CV | 6.8 | 9.3 | 3.0 | 9.0 | 4.5 | 10.1 |
| Low control | | | | | | |
| Mean | 19.3 | 5.5 | 50.3 | 59.3 | 50.2 | 39.5 |
| SD | 1.5 | 0.1 | 3.1 | 5.2 | 3.0 | 2.6 |
| CV | 7.8 | 2.2 | 6.2 | 8.8 | 6.0 | 6.6 |
| Interassay results | | | | | | |
| High control | | | | | | |
| Mean | 7,222.3 | 639.0 | 13,653.2 | 15,359.5 | 14,364.9 | 6,078.8 |
| SD | 820.1 | 79.4 | 1,831.2 | 1,429.0 | 664.6 | 690.0 |
| CV | 11.4 | 12.4 | 13.4 | 9.3 | 4.6 | 11.4 |
| Medium control | | | | | | |
| Mean | 329.8 | 40.5 | 701.3 | 884.7 | 708.4 | 534.4 |
| SD | 44.4 | 11.0 | 105.6 | 105.7 | 49.0 | 110.0 |
| CV | 13.5 | 272 | 15.1 | 12.0 | 6.9 | 20.6 |
| Low control | | | | | | |
| Mean | 34.1 | 2.7 | 71.7 | 80.5 | 69.7 | 62.3 |
| SD | 6.2 | 2.6 | 21.3 | 19.4 | 21.3 | 19.0 |
| CV | 18.2 | 94.3 | 29.7 | 24.1 | 30.6 | 30.5 |

IFN, interferon; IL, interleukin.
* Mean and SD values are given in picograms per milliliter. The coefficients of variations (CV) are given as percentages.

### Cytokine Responses of Patients and Neonates With Hyperimmunoglobulinemia E Syndrome

To validate the clinical usefulness of the 6-cytokine multiplexed assay, cytokine concentrations in tissue culture supernatant derived from stimulated mononuclear cells of patients with hyperimmunoglobulinemia E syndrome and neonates were assessed.

The production of cytokines IL-2, IL-4, IL-6, IL-10, IL-12, and IFN-gamma by 6 patients with hyperimmunoglobulinemia E syndrome and the peripheral blood mononuclear cells of 12 control subjects in response to *S aureus*, one of the major pathogens affecting the patients, and medium alone was measured. We found no statistically significant differences in cytokine production in medium alone between patients and control subjects for any of the 6 cytokines measured. When comparing the cytokine production of patients and control subjects in response to stimulated peripheral blood mononuclear cells, the average concentrations of IL-6, IL-10, and IL-12 were lower in patients than in control subjects, but the differences were not statistically significant **Figure 2**. The average IFN-gamma production of *S aureus*–stimulated control samples (304 pg/mL) vs patient samples (20 pg/mL) was, however, statistically different using a 2-tailed, unpaired *t* test ($P = .044$) (Figure 2).

Unstimulated (medium alone) and group B streptococci–stimulated neonatal cord blood–derived mixed mononuclear cells showed no statistically significant differences in IL-2, IL-4, IL-10, IL-12, or IFN-gamma cytokine production after 48-hour culture incubations. The cord blood cells produced significant concentrations of IL-6 when stimulated with group B streptococci (mean, 30,957 pg/mL) compared with the unstimulated cells (mean, 182 pg/mL) ($P = .001$) **Figure 3**. Adult group B streptococci–stimulated peripheral blood mononuclear cells released significantly greater concentrations of IFN-gamma, IL-12, and IL-6 compared with unstimulated adult cells (Figure 3). After 48-hour culture incubations, the average IFN-gamma production for unstimulated adult cells was 0 pg/mL compared with 118 pg/mL for group B streptococci–stimulated adult cells and was statistically significant ($P = .017$). For IL-12, the average cytokine production for unstimulated adult cells was 6.7 pg/mL compared with 51.3 pg/mL for group B streptococci–stimulated adult cells and was statistically significant ($P = .007$; 2-tailed, unpaired *t* test). The average IL-6 production for unstimulated adult cells was 317 pg/mL compared with 19,789 pg/mL for group B streptococci–stimulated adult cells and was statistically significant ($P = .0003$).

Neonatal group B streptococci–stimulated cells also produced significantly less IFN-gamma and IL-12 than did the stimulated adult cells. The average IFN-gamma cytokine production for neonatal cells was 1.2 pg/mL compared with 118.6 pg/mL for the adults' group B streptococci–stimulated cells ($P = .017$). Similar results were observed with IL-12

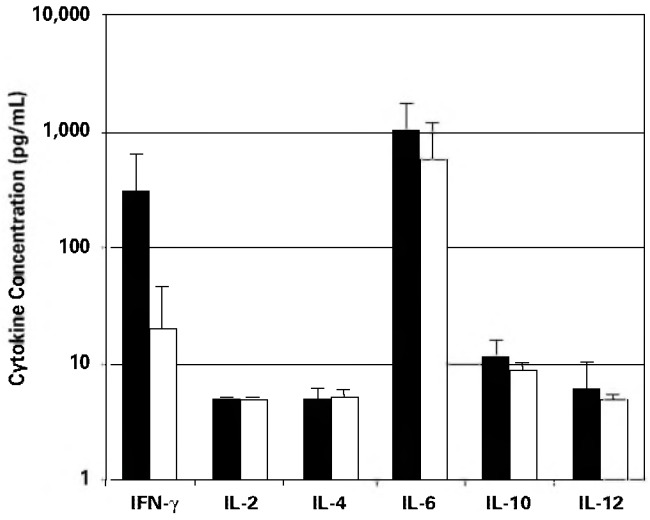


**Figure 2** Mean cytokine production by mixed peripheral blood mononuclear cells of 12 healthy control subjects (black bars) and 6 patients with hyperimmunoglobulinemia E syndrome (white bars) in response to *Staphylococcus aureus* showing a significant decrease in interferon (IFN)-gamma production ($P = .044$) in patients vs control subjects. IL, interleukin.

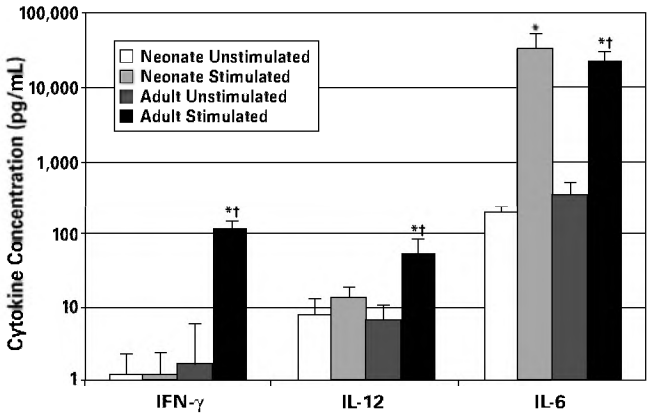


**Figure 3** Mean interferon (IFN)-gamma, interleukin (IL)-2 and IL-6 cytokine production by mixed mononuclear cells of 8 adult control subjects and 8 neonates in response to group B streptococci. Results show no significant IFN-gamma and IL-12 production in stimulated vs unstimulated neonatal cells and significantly less IFN-gamma and IL-12 production in stimulated neonatal vs adult cells. * $P < .05$, stimulated vs unstimulated cells. † $P < .05$, adult stimulated vs neonatal stimulated cells.

production, in which the stimulated neonatal cells produced significantly less cytokine than did the adults' stimulated cells (13.5 vs 51.3 pg/mL; $P = .017$; Figure 3).

## Discussion

An assay with a large dynamic range is needed for measuring cytokine concentrations because of the varied and widely ranging cytokine responses generated by antigen- and mitogen-stimulated peripheral blood mononuclear cells of immunodeficient patients and healthy control subjects. This is especially true when working with tissue culture supernatants in which the sample volume usually is insufficient for multiple dilutions, and repeated assay runs often are required to determine a final result for samples having high cytokine concentrations.

Standard curves developed by diluting recombinant cytokines over a 4- to 5-logarithmic range of concentration produced a reportable range of less than 10 to 50,000 pg/mL for all 6 multiplexed cytokines. To generate this large dynamic range, washing of the microspheres between reagent additions was necessary to reduce background fluorescence to achieve maximum sensitivity. The use of a 5-parameter curve fit also contributed to the large dynamic range. By conducting linearity studies using spiked recombinant cytokine samples and peripheral blood mononuclear cell–stimulated patient samples, both the high end (up to 30,000 pg/mL) and low end (down to 14 pg/mL) of the reportable range of the assay was studied. This also ensured that the assay could accurately quantitate both recombinant and native human cytokines. Mean ± SD recoveries for the recombinant cytokines in the multiplex assay were good (104% ± 17%) and ranged from a low of 79% for IL-12 to a high of 126% for IL-6 (Table 1). These findings were very similar to published results of Kellar et al,[17] who reported percentages of recovery ranging from 79 to 122 for 2 multiplexed assays for the quantitation of 4 cytokines each.

In our assay, the cytokine with the lowest percentage of recovery (IL-12) also showed the highest amount of cross-reactivity with IFN-gamma (Table 2). Percentage of recovery results for the lower end of the reportable range of the assay using native cytokines from tissue culture supernatant were similar to our recombinant spiked sample studies. Recoveries ranged from 88% for IL-10 to 131% for IFN-gamma, with an overall mean ± SD of 101% ± 17%. The sensitivity or detection limit for the 6-cytokine multiplexed assay was less than 10 pg/mL for all cytokines, with a mean ± SD of 5.1 ± 1 pg/mL. These sensitivities are equivalent to those of most ELISAs and were lower than those reported by Kellar et al[17] for their Luminex-based, 4-cytokine multiplexed assays. For the same 6 cytokines, Kellar et al[17] reported an average sensitivity of 10.8 pg/mL for their low sensitivity (1,000-bead) assay. The increase in sensitivity of the assay described herein is probably due to the washing steps used in our protocol, which remove any unbound or excess cytokine, conjugate, and fluorescent reporter that can increase background signal. In experiments in which the same multiplexed assay was run without washing, the fluorescence intensity values for the high standard (50,000 pg/mL) were decreased by up to 58%, while the 0 cytokine standard (background) had up to a 531% increase in fluorescence intensity units compared with the washed assay results (data not shown). The use of washing steps also reduces the amount of initial optimization required to obtain the correct concentration of capture antibodies, biotinylated secondary antibodies, and reporter fluorochrome, and reoptimization is not required between preparations of coupled microspheres. A disadvantage of washing is that it increases manual labor by 12 to 15 minutes per assay run and the reagent cost by $9 for the Millipore 96-well filtration plate.

Owing to the multiplexed format of this assay, in which multiple cytokines and capture and detection antibodies are present in the same reaction mixture, cross-reactivity becomes a much greater issue than with ELISAs, in which the measurement of each cytokine is done in separate reaction wells. For the development of a sensitive and specific assay, the selection of good antibody pairs is, therefore, crucial. Fortunately, there are many commercial vendors offering "matched" antibody pairs designed specifically for ELISA development. In our experience, these matched antibody pairs generally have worked well when used with the Luminex platform. A notable exception was in the development of the IL-2 assay, in which the best specific signal/background ratios were obtained using the BD Biosciences capture antibody, recombinant IL-2 from R&D Systems, and the Pierce-Endogen secondary antibody.

Results from the Luminex instrument showed a high degree of precision in both intra-assay and interassay studies (Table 3). In the multiplexed cytokine assay, the Luminex instrument was set to count 200 microspheres of each cytokine, for a total of at least 1,200 analyzed microspheres for each reaction well. The final reported result (in pg/mL) is, therefore, the median value of at least 200 analyzed microspheres for each cytokine. Since each individual microsphere is in essence its own immunoassay, this would be analogous to testing each individual patient sample in replicates of 200 in an ELISA for each individually measured cytokine. It is this measure of redundancy that gives the Luminex instrument its high level of precision.

The clinical usefulness of the 6-cytokine multiplexed assay was demonstrated by measuring cytokine concentrations in tissue culture supernatant derived from stimulated mononuclear cells of immunodeficient patients and neonates. The *S aureus*–stimulated peripheral blood mononuclear cells

from 6 patients with diagnosed hyperimmunoglobulinemia E syndrome had significantly lower IFN-gamma production than samples from healthy control subjects. These observations agree with those of Del Prete et al[7] and Borges et al,[2] who found that cells from patients with hyperimmunoglobulinemia E syndrome failed to make adequate amounts of IFN-gamma. IFN-gamma and IL-12 production by mixed mononuclear cells derived from neonatal cord blood was significantly less than that of adult cells when stimulated with group B streptococci. These observations are consistent with reports by Joyner et al,[8] Bryson et al,[9] and Wilson et al,[10] who reported deficient IFN-gamma and IL-12 production by neonates. Analysis of TH1- and TH2-type cytokine responses by the mononuclear cells of patients with these immunodeficiency syndromes suggests a role for imbalances in the cytokine repertoire in selected diseases. These studies demonstrate the potential of cytokine measurement in the clinical laboratory as a diagnostic tool for assessing immunodeficiencies or diseases known to result in depressed or defective cytokine production.

The multiplexing capability of the Luminex instrument is invaluable for the development of multiple analyte profiles that require smaller amounts of patient samples and reagents and less cost than traditional diagnostic methods. We found the Luminex instrument to be an accurate and reliable system for simultaneously quantitating 6 cytokines from only 100 µL of patient tissue culture supernatant. Because of the efficiency and lower costs of the multiplexed assay, cytokine testing is now more feasible to perform in a clinical laboratory setting. Studies are increasingly showing correlations between cytokine production and immunologic inflammatory disorders and other disease processes. With the ability to measure multiple cytokines from a single sample source, we have a powerful research and clinical tool for assessing cytokines produced in cellular responses.

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